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ARTICLE

Validation of a Cell-Free Translation Assay for Detecting Shiga Toxin 2 in Bacterial Culture

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A cell-free translation (CFT) assay for detecting Shiga toxin (Stx) at different levels of purity has been validated. The limits of detection for pure Stx2 (PStx2) and partially pure Stx2 (PPStx2) in water reached 20 and 3.5 pg/μL, respectively, without the artificial process of proteolytic activation and reduction of the pro-toxin. The specific detection of Stx2 was confirmed by a neutralization test using Stx2-specific mouse monoclonal antibody. This assay can be used for differentiation of Stx-producing *Escherichia coli* from non-Stx-producing *E. coli*. Four *E. coli* O157:H7 strains genotypically different for Stx were tested. The translational inhibition of Stx-producing *E. coli* was significantly higher than that of non-Stx-producing *E. coli* when bacterial culture supernatants were used for the analysis. Inhibition occurred even with supernatants diluted 1000-fold. The thermal stability of Stx2 was studied using the CFT assay, and significant differences were observed among three Stx2 preparations heated at 70 °C for 60 min. It was concluded that the CFT assay is a rapid, specific, and sensitive method for detecting Stx2 activity.

KEYWORDS: Cell-free translation assay; *E. coli* O157:H7 strains; limit of detection; Shiga toxin 2; thermal stability of Stx2

INTRODUCTION

Shiga toxin (Stx) of enterohemorrhagic *Escherichia coli* (EHEC) was first described in 1983 by O'Brien et al. (*1*), who reported that *E. coli* O157:H7, the causative agent of hemorrhagic colitis (*2*), produced high levels of cytotoxin active against Vero cells that could be neutralized by rabbit antibody against Stx. Two types of cytotoxins have been identified subsequently among *E. coli* O157:H7 strains designated Stx1 and Stx2 (*3*). Although both toxins are bacteriophage encoded (*4*, *5*) and have similar molecular structures and biological activities, they share only 60% deduced amino acid sequence similarity (*6*) and are distinct antigenically (*3*). Studies with the prototype Stx indicate that the holotoxin consists of an A subunit (MW 32000) and a pentamer of B subunits (MW 7700 each) (*7*, *8*). The A subunit is responsible for *N*-glycosidase activity, which removes a single adenine residue from the 28 S rRNA and disrupts protein synthesis (the same mode of action found in the plant toxin, ricin) (*9*), whereas the B subunit is responsible for the binding of toxin to the eukaryotic cell receptor, globotriaosylceramide or Gb3 (*10*), which determines the specificity of the toxin for target cells. It was reported that for in vitro activity, the holotoxin required activation by trypsin, urea, and reducing agents for removal of the B subunits and release of the enzymatically active A1 fragment (MW 28000) from the A subunit (*8*).

It is now clear that the Stx of *E. coli* is one of the most important virulence factors in the pathogenesis of certain diarrhea diseases, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (*11*−*13*). Although *E. coli* strains from several serotypes are capable of producing Stxs, most outbreaks of hemorrhagic colitis and the HUS investigated have been associated with serotype *E. coli* O157:H7 (*14*). Epidemiological studies have indicated that Stx2 may be a more important determinant of pathogenicity than Stx1 (*15*, *16*).

Currently, detection of Stx-producing bacteria is performed by cell culture toxicity assays that are expensive, time-consuming, and require specialized facilities and a level of expertise not generally available in clinical laboratories, particularly those in developing countries. Immunochemical assays such as ELISA and colony immunoblot assays have played an important role in screening of Stx-producing *E.coli*. They typically take 3−24 h, and the specificity and sensitivity of these assays rely chiefly on the antibodies. These assays are unable to distinguish active from inactive toxins. Recently, a cell-free translation (CFT) assay was developed for measuring the biological activity of ribosome-inactivating proteins (RIP) based on their ability to inhibit protein synthesis using luciferase as a marker (*17*). The method was demonstrated to be specific, precise, and accurate for measuring ricin activity (*18*). We present in this study our results on the application of the CFT assay for the detection of Stx2 without the usual requirements for in vitro activation and reduction of the toxin. The assay was validated for detecting Stx2 in bacterial cultures. The thermal stability of Stx2 at different levels of purity was also investigated using this assay.

MATERIALS AND METHODS

Source of Toxins. Pure Stx2 (PStx2) was purchased from List Biological Laboratories, Inc. (Campbell, CA). Briefly, the toxin was

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isolated from *E. coli* O157:H7 and purified by affinity chromatography with the glycolipid receptor Gb3. The toxin was packaged at 0.1 mg/mL in 0.1 M Tris, pH 8.6, 0.1 M NaCl, and 0.001% polyvinyl alcohol. Partially pure Stx2 (PPStx2) was purchased from Toxin Technology, Inc. (Sarasota, FL). Briefly, the preparation was purified from a recombinant strain containing a cloned gene for Stx2 on the plasmid pJES. *E. coli* C-600 cells harvested from the overnight culture were broken by sonication, and the supernatant was collected after centrifugation and processed through two ion-exchange chromatography columns and one gel filtration column. The final preparations contained about 25% Stx2 as measured by ELISA.

Bacterial culture supernatants were prepared as described previously (*19*) from the following *E. coli* O157:H7 strains: RM1697 (*20*) (cow fecal isolate; strain 42) containing both Stx1 and Stx2 genes (+/+), RM6039 (*21*) (human; spinach outbreak, 2006) containing Stx2 gene only (−/+), RM1625 (*20*) (cow fecal isolate; strain 33) containing Stx1 gene only (+/−), and RM4876 containing no Stx genes (−/−). Bacterial cells were grown overnight in Luria−Bertani (LB) liquid medium at 37 °C to an optical density at 600 nm (OD_{600}) of 1.86. Following centrifugation at 13000*g* for 10 min at 4 °C, the supernatants were collected and filtered through a 0.2 μm filter to remove intact cells and other debris. The amounts of Stx2 in the filtered culture supernatants were estimated by ELISA. The biological activities of the Stx2 in the supernatants were examined by CFT assays.

Toxicity Assay for Stx2. Serial dilutions of Stx2 from different preparations were added to the translation lysate mixture consisting of nuclease-treated rabbit reticulocyte lysate (catalog no. L4960), complete amino acid mixture (1 mM, catalog no. L4461), RNasin ribonuclease inhibitor (40 units/μL, catalog no. N2111), nuclease-free water (catalog no. P1193), and luciferase mRNA (1 mg/mL, L4561) in a ratio (v/v) of 35:1:1:36:2, respectively. All components were purchased from Promega (Madison, WI). The ratio of Stx2 and translation lysate mixture was 1:5 (v/v), and the conditions for the CFT assay were as previously described (*22*). The Bright-Glo Luciferase Assay System was purchased from Promega (catalog no. E2620). Black microtiter plates (NUNC 96-well Maxisorp) were purchased from Fisher Scientific Inc. (Pittsburgh, PA). Luminescence was measured as counts per second (cps) in a Victor II plate reader (Perkin-Elmer, Shelton, CT). Translation lysate mixture with water or LB in lieu of Stx2 was used as a negative control. Toxin activity was calculated as the percentage of inhibition of translation [(cps in negative control − cps in Stx2-treated sample)/cps in negative control] × 100. All data represent the mean ± standard deviation (SD) of triplicate samples measured in a representative experiment. Three individual experiments were performed. Small variations in results between experiments were observed due to slight changes of toxin activity and other conditions. Standard curves were plotted for values of inhibition versus the log concentration of Stx2.

Limit of Detection (LOD). The LOD was defined as the concentration corresponding to a signal 3 times the noise level of the negative control. Alternatively, if the LOD calculated by this method fell below the concentration of the most dilute standard used to determine the calibration curve, the LOD would be set to the concentration of the most dilute standard.

Neutralization Test. Toxin activity in PStx2 and PPStx2 preparations was neutralized by mixing serial dilutions of the mouse monoclonal antibody (mAb) IgG against Stx2A, Sc-65471 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), with an equal volume of a constant dilution of Stx2 containing approximately 4 times the IC_{50} of the toxin (concentration of toxin giving 50% inhibition of protein synthesis). The mixture was incubated at 37 °C for 1 h, and 3 μL then was added to 15 μL of translation lysate mixture to measure residual toxicity assessment as described previously (*22*). The relative activities of the samples were calculated by normalizing the values to the activity of Stx2 samples without neutralization by mAb as 100%.

Quantification of Stx2 in PPStx2 Preparation and Cell-Free Culture Supernatants. ELISA was used to determine the amount of Stx2 present in the PPStx2 preparation and bacterial culture supernatants. Capture antibody against Stx2, clone VT135/6-B9 (Sifin, Berlin, Germany), was diluted at 1:300 in PBS, and 100 μL was added to wells of a 96-well plate overnight at 4 °C. The wells were washed and the plate was blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS) for 1 h and then incubated with serial dilutions of Stx2 standard, PPStx2, and bacterial culture supernatants for 2 h. The plate was washed four times with water and then incubated for 1 h with a 1:200 dilution of horseradish peroxidase-conjugated Stx2 antibody (STX-PC, Toxin Technology, Inc.). Enhanced K-blue TMB substrate (Neogen Corp., Lexington, KY) was added to detect bound conjugated antibody.

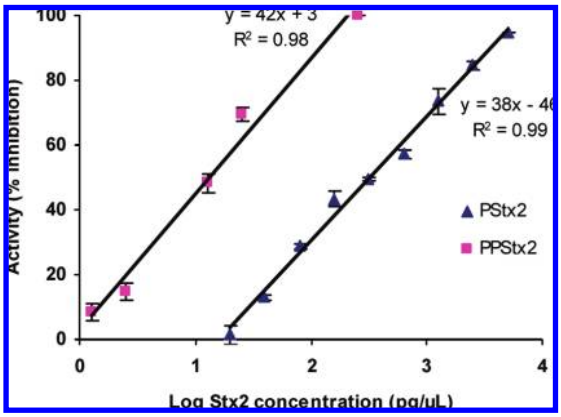

Figure 1. Activity (percent translational inhibition) versus log concentration of PStx2 and PPStx2. The activities of Stx2 were measured with the cell-free translation (CFT) assay. Results represent the mean ± SD of three replicates from one representative experiment. Three individual experiments were performed. Equations were obtained by linear regression analysis.

Table 1. IC_{50} Values and LOD of PStx2 and PPStx2

Stx2	IC_{50} (pg/μL)	LOD (pg/μL)	no. of samples
PStx2	324	20	3
PPStx2	13	3.5	3

Thermal Inactivation of Stx2. Aliquots of Stx2 samples (100 μL) were preheated at 50, 70, and 90 °C in microcentrifuge tubes for up to 60 min in a Thermomixer R (Eppendorf, Westbury, NY) and then cooled in an ice bath before the activity assay. The concentrations used for the PStx2 and PPStx2 were at their IC_{50}, and bacterial culture supernatant was at 1:10 dilution in LB. The relative activities of Stx2 were calculated by normalizing against the activity of Stx2 without heat treatment as 100%.

RESULTS

Validation of the CFT Assay for Detecting Stx2 Activity. To determine whether the CFT assay can be used to evaluate the biological activity of Stx2 omitting the process of activation, the translation lysate mixture was directly treated with serial dilutions of PStx2 without preactivation. As shown in **Figure 1**, PStx2 was readily detected and the inhibition of protein synthesis by PStx2 was log-linear and dose-dependent between 1.3 (20 pg/μL) and 3.7 (5000 pg/uL). The IC_{50} calculated from the linear regression equation of the fractional activity and log of toxin concentration was 324 pg/μL and the LOD was 20 pg/μL (**Table 1**). The average luminescence levels measured from the luciferase assays were 146647 cps for the negative control (no toxin added) and 210 cps for samples with 100% of inhibitory activity. To compare the toxicities of Stx2 at different purities, PPStx2 preparation (Toxin Technology) was analyzed. The toxin content of the PPStx2 preparation was determined to be approximately 25% when measured by ELISA (data not shown). As indicated in **Figure 1**, the inhibition curve of the PPStx2 in water was linear between 0.1 and 2.4 (1.25 and 250 pg/μL). The IC_{50} was 13 pg/μL and the LOD was 3.5 pg/μL (**Table 1**). Thus, the LOD and IC_{50} values for the PPStx2 were approximately 5.7- and 25-fold lower, respectively, compared to the PStx2.

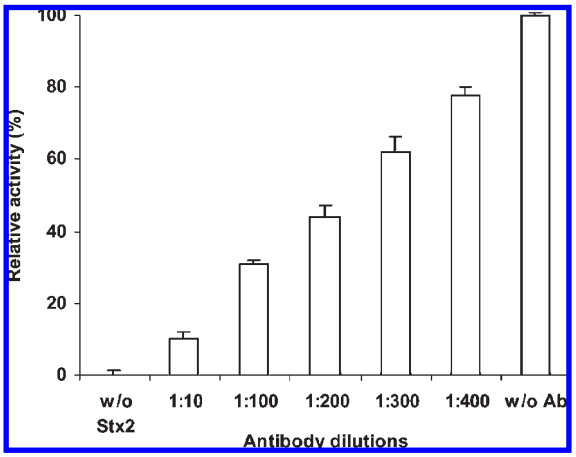

Figure 2. Neutralization of PPStx2 activity by mouse mAb (IgG) against Stx2A (Sc-65471). Serial dilutions of mAb were incubated with an equal volume of Stx2 at 4IC_{50} (52 ng/μL of actual Stx2) or 1:10 dilution of mAb in the absence of Stx2 as labeled (w/o Stx2) for 1 h at 37 °C before addition to the translation assay. Luminescence was measured after 90 min of incubation at 30 °C. The relative activity was calculated by normalizing against the activity of Stx2 without neutralization as 100%. Results represent the mean ± SD of three replicates from one representative experiment. Three individual experiments were performed.

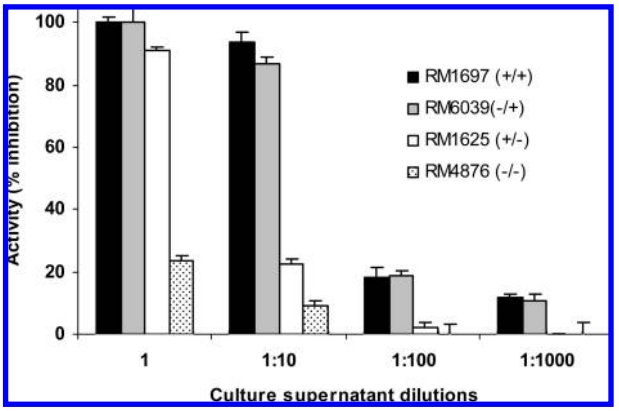

Figure 3. In vitro inhibition of bacterial culture supernatants in protein translation. The inhibitory activities of culture supernatants from bacterial strains RM1697 (+/+), RM6039 (−/+), RM1625 (+/−), and RM4876 (−/−) were measured with the CFT assay. Results represent the mean ± SD of three replicates from one representative experiment. Three individual experiments were performed.

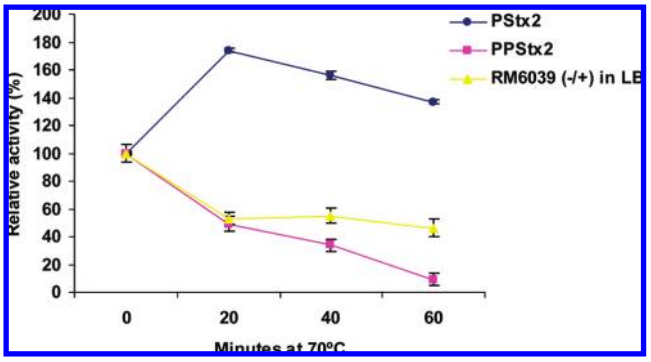

Figure 4. Thermal inactivation curves for PStx2, PPStx2, and culture supernatant of bacterial strain RM6039 (−/+) at 70 °C. For PStx2 and PPStx2, the IC_{50} concentration was used; for bacterial culture supernatant, a 1:10 dilution in LB medium was used for heat treatment. The relative activity was calculated by normalizing against the activity of Stx2 without heat treatment as 100%. Results represent the mean ± SD of three replicates from one representative experiment. Three individual experiments were performed.

To confirm that the translational inhibition observed in these assays is Stx2 specific, neutralization tests were performed by incubating samples with Stx2 specific antibody before the CFT assay. **Figure 2** shows the effect of Stx2 antibody (Sc-65471) on the toxicity of PPStx2 at four IC_{50} values. The relative activity of each sample was calculated by normalizing the values to the activity of Stx2 samples without neutralization by mAb as 100% (the corresponding luminescence reading measured was about 38069 cps). Over 90% of the enzyme activity was abolished when a high concentration of neutralizing antibody was used and the average luminescence reading reached around 175119 cps (**Figure 2**, 1:10 dilution). Antibody at 1:10 dilution in the absence of Stx2 had no effect on protein translation, and the average luminescence reading was around 190347 cps (**Figure 2**, w/o Stx2). The preincubation of PPStx2 with a nonspecific IgG mAb did not neutralize the toxicity of Stx2 (data not shown). The Stx2-specific mAb also effectively (90%) neutralized the activity of the PStx2 in the CFT assay (data not shown).

Identification of Stx-Producing *E. coli* by the CFT Assay. To determine whether the CFT assay can be used for differentiating Stx-producing *E. coli* from non-Stx-producing *E. coli*, four pathogenic *E. coli* O157:H7 strains from different sources and known Stx gene profiles, RM1697 (+/+), RM6039 (−/+), RM1625 (+/−), and RM4876 (−/−), were examined by this method. The production of Stx by bacteria was evaluated on the basis of the ability of bacterial culture supernatant to inhibit protein synthesis. The LB medium was used as a negative control. The average luminescence levels corresponding to the negative control (no inhibition) and full activity (100% translational inhibition) were 235143 and 110 cps, respectively. **Figure 3** shows the translational inhibition of protein synthesis by various bacterial supernatants. The order of inhibition level from high to low was strains RM1697 (+/+), RM6039 (−/+), RM1625 (+/−), and RM4876 (−/−). Statistical analysis of differences between strains indicated that the inhibitory activity of the −/− strain was significantly less than those of strains containing one or both Stx genes ($p < 0.0001$); the activity of the Stx2 strain, RM6039 (−/+), was significantly higher than that of the Stx1 strain, RM1625 (+/−) ($p < 0.0001$) when using undiluted and diluted culture supernatant up to 100-fold. The CFT assay distinguished Stx2-producing *E. coli* strains, RM1697 (+/+) and RM6039 (−/+), from the −/− strain (RM4876) even when the culture supernatant was diluted 1000-fold ($p < 0.0001$). The results clearly correlated with the Stx profile. A low level of inhibitory activity was observed in the supernatant of the −/− strain compared with the negative control, LB medium (**Figure 3**). To confirm the association of the Stx profiles based on gene content with the production of Stx, ELISA was performed using the PStx2 as standard. Stx1 was not measured due to the unavailability of a standard. The amounts of Stx2 were estimated to be 580 and 550 ng/mL in the supernatants of bacterial strains, +/+ and −/+, respectively. There was no detectable Stx2 in supernatants of bacterial strains +/− and −/− using mAb VT135/6-B9 as a capture antibody.

The CFT Assay Can Be Used To Study the Thermal Stability of Stx2. To expand the use of the CFT assay, the thermal stability of Stx2 was studied (**Figure 4**). Toxins at different levels of purity were heated at 50, 70, and 90 °C for up to 60 min. For pure and partially pure toxin preparations a final concentration of IC_{50} was tested; for the bacterial culture supernatants 1:10 dilutions in LB were measured. It was found that there was no loss of toxin

activity for three toxin preparations heated at 50 °C for up to 60 min, but total loss of activity was found in toxin preparations heated at 90 °C for 20 min (data not shown). The thermal inactivation curves obtained from three Stx2 preparations were very different at 70 °C. For the PStx2, the enzyme activity increased up to 74% after heating for 20 min, whereas for the PPStx2, the enzyme activity decreased rapidly and only 50% of the activity remained after being heated for 20 min; by 60 min $<20\%$ of activity was detected. For the bacterial culture supernatant, 50% of toxin activity was lost in the first 20 min and minimal loss of activity occurred thereafter. In these experiments, the actual luminescence readings corresponding to the negative control (without Stx2) and samples with 100% relative activity were 179000 and 87487 cps, respectively.

DISCUSSION

The major results of this study were the demonstration that the CFT assay is feasible for detection of biologically active Stx, useful for screening of Stx-producing *E. coli* and for measurement of the thermal stability of Stx. Compared with other detection methods, this assay has the advantage of detecting both the presence and the activity of Stx. It is also faster and easier as the entire assay can be accomplished in 90 min. Early work (*23*) reported that full in vitro activity of Stx required both removal of the B subunits and activation of the A subunit by treatment of the holotoxin with trypsin, urea, and dithiothreitol. Our results demonstrated that Stx2 can be detected without the tedious and time-consuming process of activation, and the LOD for nonprocessed Stx2 is close to the reported LOD for preactivated Stxs (*23*) using the CFT assay. This result suggests that either the pro-toxin of Stx2 is active or the pro-toxin is preactivated in our experimental conditions. This finding is important because it shortens the assay time and makes the assay more practical for detection of Stxs in biological samples.

A log-linear inhibition was observed for both pure and partially pure Stx2 within the ranges indicated in **Figure 1**. Similar to our studies of ricin (*22*), we observed that the PStx2 inhibited protein translation less than did the PPStx2. This may be due to the PPStx2 preparation being less denatured because of fewer purification steps or there existed toxin stabilizers in the PPStx2 preparation.

With the increase of human illness outbreaks caused by Stx-producing *E. coli* O157:H7 (*24*), there is a critical need for identification of the reservoirs and effective control of the pathogen. The CFT assay described can rapidly differentiate Stx-producing *E. coli* from non-Stx-producing *E.coli* using bacterial culture supernatant. It was found that the bacterial strain, an Stx2 strain, RM6039 (−/+), caused higher inhibition of protein translation consistently compared to the Stx1 strain, RM1625 (+/−). However, it is unknown whether this is due to the difference in the amounts of toxin expressed or differences in the relative toxicities of Stx1 and Stx2 in the CFT assay. Our ELISA result indicates that the amount of Stx2 in the −/+ strain supernatant was 550 ng/mL; however, the amount of Stx1 in +/− strain supernatant was not determined due to the lack of a PStx1 standard. It was reported that Stx1 is often predominant in cell lysates, whereas Stx2 is present in much higher titer than Stx1 in culture supernatants (*25*). Head et al. reported that Stx1 and Stx2 displayed indistinguishable protein inhibition activity in a cell-free system (*26*). The higher inhibition could result from the higher titer of Stx2 in the −/+ strain supernatant. **Figure 3** shows that the undiluted supernatant of bacterial strain −/− exhibited 20% inhibition in the CFT assay when using LB medium as a negative control, even though no Stx1 or Stx2 gene products were identified by PCR or ELISA. We speculate that some components or another toxin of unknown type in the bacterial culture supernatant may account for the low activity in the CFT assay. Despite this, the CFT assay is still a useful tool for initial screening of Stx2-producing *E. coli* because the difference in translational inhibition between Stx2-producing and non-Stx2-producing *E. coli* is significant (**Figure 3**). Alternatively, to reduce the background noise, instead of using the LB medium, the supernatant of a −/− strain could be used as the negative control for Stx2-producing *E. coli* by the CFT assay.

Heat treatment is one of the fundamental strategies to inactivate toxins. Studies on heat inactivation of *E. coli* O157:H7 have demonstrated that the temperature required by the FDA for the cooking of ground beef (71 °C) is adequate to inactivate the bacteria (*27*). However, whether the same temperature could inactivate the toxins produced by *E. coli* has not been thoroughly studied. Results obtained in this study indicate that the thermal stabilities of Stx2 at different levels of purity could be very different. The toxicity of the PStx2 increased dramatically after being heated at 70 °C for 20 min, whereas at the same condition, the activity of the PPStx2 decreased significantly. This may be explained by the holotoxin unprotected by other components unfolding by heat, resulting in a more active enzyme. Brigotti et al. reported that heat treatment at acidic pH stimulated a burst in the enzymatic activity of the nonactivated pro-enzyme (*28*). It is not known what caused the variations in enzyme activity and thermal stability among different Stx2 preparations. These differences could reflect differences in the purity, physiological state, and extent of denaturation of toxin preparations. Nevertheless, the CFT assay is a specific, sensitive, and rapid method for measuring the toxicity of PStx2 and PPStx2. It has the capacity to be used for high-throughput screening of Stx inhibitors and detecting Stx-producing *E. coli*.

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